REMARKS

Status of the Claims

Claims 1-4, 7, 9, 22, 24, 26, 28, 30, 31, 37, 41-43, 46, 47, 49, 53, 57-61 and 65-76 are currently pending. Claims 65-76 were withdrawn from further consideration under 37 CFR § 1.142(b) as being drawn to a nonelected invention. Claims 1-4, 7, 9, 22, 24, 26, 28, 30, 31, 37, 41-43, 46, 47, 49, 53 and 57-61 were examined and rejected. Claim 1 has been amended. Support for this amendment may be found, for example, in the specification at paragraphs **[0027]**, **[0066]**, **[0071]** and **[0149]** and in original claims 40 and 47 (*see* US 2007/0134661 A1, published Jun. 14, 2007). Thus, no new matter has been introduced by way of this amendment. Claim 47 has been canceled. Upon entry of the amendment, claims 1-4, 7, 9, 22, 24, 26, 28, 30, 31, 37, 41-43, 46, 49, 53, 57-61 and 65-76 will be pending. Entry of the amendment and reconsideration in view of the following comments is respectfully requested.

With respect to all amendments, Applicants have not dedicated or abandoned any unclaimed subject matter and moreover have not acquiesced to any rejections and/or objections made by the Patent Office. Applicants expressly reserve the right to pursue prosecution of any presently excluded subject matter or claim embodiments in one or more future continuation and/or divisional application(s).

Objection to Specification

The specification disclosure was objected to for allegedly failing to comply with the requirements of 37 CFR §§ 1.821-1.825 because the specification failed to identify nucleotide sequences in Table 1 by SEQ ID numbers which could be matched with the SEQ ID numbers previously submitted in a computer readable format to the USPTO. Since Applicants have amended Table 1 of the specification to include the requisite SEQ ID NOs (and to correct obvious typographical errors at SEQ ID NOs: 74 and 75 (removing extra spaces) and at SEQ ID NO: 213 (replacing a B with the correct nucleotide G), this objection may be withdrawn.

Additionally, due to the inadvertent omission of a number of sequences from the previously filed sequence listing, Applicant submits herewith a substitute sequence listing in ASCII .txt format via EFS-Web in compliance with 37 C.F.R.§1.821(c) and §1.8215(a) and (b). No new matter has been added. Accordingly, entry of the substitute sequence listing into the above-captioned application is respectfully requested.

Rejections under 35 U.S.C. § 102

Anticipation by Apple

Claims 1, 3-4, 9, 22, 24, 28, 30-31, 37, 49, 53 and 61 were rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Apple et al. (US 5,567,809, hereinafter "Apple"). The Office alleges that Apple teaches a method of HLA DR beta DNA (DRB) typing, which includes preparing the target nucleic acids from cell lines, and further teaches using genomic DNA that contains other genes not related to HLA DR beta. Apple further allegedly teaches a reverse dot blot (chip), and further teaches that the chip contains probes specific for particular allele type and additionally contains a control probe that detects all of the alleles. The control probe of Apple is allegedly the positive control probe as defined in the instant specification and also because it detects all of the DRB alleles. Finally, Apple allegedly teaches hybridizing the array and assessing hybridization between said target nucleotide sequence and/or said another nucleotide sequence and said probes comprised on said chip to determine the type of DRB target gene.

Applicants respectfully traverse this rejection. The legal standard for anticipation under 35 U.S.C. § 102 is one of strict identity. *Trintec Industries, Inc. v. Top-U.S.A. Corp.*, 63 U.S.P.Q.2d 1597 (Fed. Cir. 2002). To anticipate a claim, a single prior source must contain each and every limitation of the claimed invention. *In re Paulson*, 30 F.3d 1475, 1478-79, 31 USPQ2d 1671, 1673 (Fed. Cir. 1994) (citing *In re Spada*, 911 F.2d 705, 708, 15 USPQ2d 1655, 1657 (Fed. Cir. 1990)). "A claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Bros. v. Union Oil Co. of California*, 814 F.2d 628, 631, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987); MPEP § 2131.

As an initial matter, claim 1 has been amended to require that the chip comprise all four immobilized control probes: a positive control probe, a negative control probe, a hybridization control probe and an immobilization control probe. Claim 1 has been further amended to recite the following limitation: "multiple positive control probes are immobilized on the chip, and the variations in the length and sequence of the immobilized positive control probes, when hybridized with the target nucleotide sequence or the another nucleotide sequence in the preparation provided in step a), create a group of hybridization signals having strong-to-weak or weak-to-strong orderly magnitude." Since all of claims 3-4, 9, 22, 24, 28, 30-31, 37, 49, 53 and 61 depend, directly or indirectly, from independent claim 1, each of those claims also incorporates the newly added limitations.

Apple does not disclose a chip comprising all four immobilized control probes, or a chip comprising multiple immobilized positive control probes, wherein the variations in the length and sequence of said probes, when hybridized with the target nucleotide sequence or another nucleotide sequence in the preparation, create a group of hybridization signals having strong-to-weak or weak-to-strong orderly magnitude. Since Apple fails to teach each and every limitation of the claimed invention, the strict identity standard for anticipation under 35 U.S.C. § 102(b) is not met. Accordingly, it is respectfully submitted that this rejection should be withdrawn.

Anticipation by Straus

Claims 1-2, 4, 9, 22, 24, 26, 41, 43, 49 and 53 were rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Straus (US 2002/0086289, hereinafter "Straus"). The Office alleges that Straus teaches a method for genomic profiling that includes obtaining samples, enriching and lysing cells, and fixing the DNA onto a solid support. Straus also allegedly teaches adding positive control DNA samples not related to test DNA samples and further teaches a detection array (chip) containing positive and negative control probes. Finally, Straus allegedly teaches hybridizing the array and assessing hybridization between the target nucleotide sequence and/or another nucleotide sequence and said probes comprised on said chip to determine the type of said target gene.

Applicants respectfully traverse this rejection. The legal standard for anticipation and the current amendment of claim 1 have been described above. Since all of claims 2, 4, 9, 22, 24, 26, 41, 43, 49 and 53 depend, directly or indirectly, from claim 1, each of those claims also incorporates the newly added limitations.

Straus does not disclose a chip comprising all four immobilized control probes, or a chip comprising multiple immobilized positive control probes, wherein the variations in the length and sequence of said probes, when hybridized with the target nucleotide sequence or another nucleotide sequence in the preparation, create a group of hybridization signals having strong-to-weak or weak-to-strong orderly magnitude. Since Straus fails to teach each and every limitation of the claimed invention, the strict identity standard for anticipation under 35 U.S.C. § 102(b) is not met. Accordingly, it is respectfully submitted that this rejection should be withdrawn.

Rejections under 35 U.S.C. § 103

Obviousness Over Apple in View of Patterson

Claims 1-2 and 7 were rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Apple in view of Patterson et al. (US 5,843,640, hereinafter "Patterson").

The teachings of Apple have been discussed above. The Office acknowledges that Apple does not teach using leukocytes as the target cells or their isolation using magnetic microbeads. To cure these deficiencies of Apple, the Office cites Patterson, which allegedly teaches the isolation of lymphocytes from PBMCs using magnetic beads and further teaches that the magnetic bead method provides highly enriched population of CD4 lymphocytes, i.e., leukocytes. The Office asserts that it would have been obvious to one of ordinary skill in the art at the time the invention was made to modify the target cell isolation method of Apple and use the magnetic bead method of Patterson with the expected benefit of obtaining highly enriched population of leukocytes from PBMCs as taught by Patterson, thus having additional sources of cells for the HLA Typing method of Apple.

Applicants respectfully traverse this rejection. The obviousness analysis under 35 U.S.C. § 103(a) requires the consideration of the scope and content of the prior art, the level of skill in the

relevant art, and the differences between the prior art and the claimed subject matter must be considered. *KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct. 1727 (2007) (*citing Graham v. John Deere Co.*, 383 U.S. 1, 17 (1966)). To establish a prima facie case of obviousness a three-prong test must be met. First, the prior art must reference must teach or suggest all the claim limitations. *In re Royka*, 490 F.2d 981, 985 (CCPA 1974). Second, there must be some suggestion or motivation, either in the references or in the knowledge generally available among those of ordinary skill in the art, to modify the reference to achieve the claimed invention. *KSR* at 1731. And third, there must be a reasonable expectation of success found in the prior art. *In re Vaeck*, 947 F.2d 488 (Fed. Cir. 1991). Moreover, critical elements of the invention as a whole which clearly distinguish the entire invention from the prior art references cannot be ignored. *Panduit Corp. v. Dennison Manufacturing Co.*, 1 U.S.P.Q.2d 1593, 1597 (Fed. Cir.), *cert. denied*, 481 U.S. 1052 (1987).

As explained above, the amended claim 1 requires that the chip must comprise all four immobilized control probes: a positive control probe, a negative control probe, a hybridization control probe and an immobilization control probe. Claim 1 further specifies that multiple positive control probes are immobilized on the chip, and the variations in the length and sequence of the immobilized positive control probes, when hybridized with the target nucleotide sequence or the another nucleotide sequence in the preparation provided in step a), create a group of hybridization signals having strong-to-weak or weak-to-strong orderly magnitude.

Applicants respectfully submit that controls play a crucial role in genetic typing studies using DNA chips or arrays. At the time of the present invention, DNA chip based allele-specific hybridization genotyping had seen a limited number of uses primarily due to the difficulty in obtaining a good signal to noise ratio in allele-specific hybridization. The specificity of discrimination between completely matched and mismatched nucleotides in hybridization was limited, especially when many different oligonucleotides needed to be analyzed under a single hybridization condition. The poor specificity compromised genotype call rates and accuracy, and it was recognized in the art that there was no easy solution to this problem, despite many efforts in the past, including the use of chips designed with high redundancies. (*See, e.g.,* Tsuchihashi et al.,

Progress in high throughput SNP genotyping, *Pharmacogenomics J.* 2002, 2:103-110, at page 105, right column, 2nd paragraph; attached as **Exhibit A**).

Applicants have addressed the problem of poor genotyping accuracy by developing a chip comprising positive, negative, hybridization and immobilization controls immobilized thereon, and further comprising multiple positive controls of different length and sequence, such that and the variations in the length and sequence of the immobilized positive control probes, when hybridized with the target nucleotide sequence or another nucleotide sequence in the preparation, create a group of hybridization signals having strong-to-weak or weak-to-strong orderly magnitude. Since each type of control probe controls a different aspect of the genotyping process, the combination of the four controls along with a range of positive controls of various hybridization strengths results in a powerful and effective quality control system that enhances the overall accuracy of genetic typing.

In light of the foregoing, it is respectfully submitted that neither Apple nor Patterson nor a combination thereof teaches or suggests using a chip comprising all four immobilized control probes, or a chip comprising multiple immobilized positive control probes, wherein the variations in the length and sequence of said probes, when hybridized with the target nucleotide sequence or another nucleotide sequence in the preparation, create a group of hybridization signals having strong-to-weak or weak-to-strong orderly magnitude. In the absence of a teaching or suggestion of each and every claim element, the cited combination fails to provide the motivation to practice the presently claimed invention. Therefore, the Office has failed to make a *prima facie* case of obviousness, and the present rejection should be withdrawn.

Obviousness Over Apple in View of Straus

Claims 1 and 41 were rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Apple in view of Straus.

The teachings of Apple and Strauss and the legal standard for obviousness have been described above. The Office acknowledges that Apple does not teach the complementarity of positive control probe to a portion of the target nucleotide sequence. To cure this deficiency of Apple, the Office cites Straus, which allegedly teaches a method for genomic profiling including an

immobilized positive control probe that hybridizes to a target control sequence, thus meeting the definition of the positive control probe as defined in the instant specification. The Office asserts that it would have been obvious to one of ordinary skill in the art at the time the invention was made to modify the target detection method of Apple by including the positive control probe complementary to a portion of target sequence of Straus with the expected benefit of confirming the false negative result due to failure to detect a signal from positive control probe as taught by Straus.

Applicants respectfully traverse this rejection for substantially the same reasons as those set forth above. Neither Apple nor Straus nor a combination thereof teaches or suggests using a chip comprising all four immobilized control probes, or a chip comprising multiple immobilized positive control probes, wherein the variations in the length and sequence of said probes, when hybridized with the target nucleotide sequence or another nucleotide sequence in the preparation, create a group of hybridization signals having strong-to-weak or weak-to-strong orderly magnitude. In the absence of a teaching or suggestion of each and every claim element, the cited combination fails to provide the motivation to practice the presently claimed invention. Therefore, the Office has failed to make a *prima facie* case of obviousness, and the present rejection should be withdrawn.

Obviousness Over Straus in View of Delenstarr

Claims 1 and 41-42 were rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Straus in view of Delenstarr et al. (US 2002/0051973, hereinafter "Delenstarr").

The teachings of Strauss and the legal standard for obviousness have been described above. The Office acknowledges that Straus does not teach using a negative control probe that has about 1-3 base pair mismatches compared to positive control probe. To cure this deficiency of Straus, the Office cites Delenstarr, which allegedly teaches a negative control probe having about 3 base pair mismatches when compared to the positive control probe. The Office asserts that it would have been obvious to one of ordinary skill in the art at the time the invention was made to modify the negative control probe of Straus for the genomic profiling and include shorter negative control probe of Delenstarr with the expected benefit of using a negative control probe of shorter length that mimics longer probe properties, yet having reduced affinity complementary target sequence as

taught by Delenstarr, thus reducing the cost of synthesizing negative control probe in the genomic profiling method of Straus.

Applicants respectfully traverse this rejection for substantially the same reasons as those set forth above. Neither Straus nor Delenstarr nor a combination thereof teaches or suggests using a chip comprising all four immobilized control probes, or a chip comprising multiple immobilized positive control probes, wherein the variations in the length and sequence of said probes, when hybridized with the target nucleotide sequence or another nucleotide sequence in the preparation, create a group of hybridization signals having strong-to-weak or weak-to-strong orderly magnitude. In the absence of a teaching or suggestion of each and every claim element, the cited combination fails to provide the motivation to practice the presently claimed invention. Therefore, the Office has failed to make a *prima facie* case of obviousness, and the present rejection should be withdrawn.

Obviousness Over Apple in View of Samartziduo

Claims 1 and 43 were rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Apple in view of Samartziduo et al. (*Life Sci. News*, 2001, 8:1-3, hereinafter "Samartziduo").

The teachings of Apple and the legal standard for obviousness have been described above. The Office acknowledges that Apple does not teach using a hybridization control probe that is complementary to a synthetic nucleotide sequence not related to the target gene. To cure this deficiency of Apple, the Office cites Samartziduo, which allegedly teaches microarray scorecard controls on the chip that includes positive and negative hybridization control probes and probes for dynamic range and ratio controls, and further teaches that these controls are YIR artificial genes and do not hybridize to human or mouse genes and the they are added as controls for hybridization. The Office asserts that it would have been obvious to one of ordinary skill in the art at the time the invention was made to modify the HLA typing method of Apple and include the hybridization control probe of Samartziduo with the expected benefit of using a better controls for validation of microarray experiments, allowing assessment of target attachment, hybridization uniformity, detection limits, dynamic range and expression ratio as taught by Samartziduo.

Applicants respectfully traverse this rejection for substantially the same reasons as those set forth above. Neither Apple nor Samartziduo nor a combination thereof teaches or suggests using a chip comprising all four immobilized control probes, or a chip comprising multiple immobilized positive control probes, wherein the variations in the length and sequence of said probes, when hybridized with the target nucleotide sequence or another nucleotide sequence in the preparation, create a group of hybridization signals having strong-to-weak or weak-to-strong orderly magnitude. In the absence of a teaching or suggestion of each and every claim element, the cited combination fails to provide the motivation to practice the presently claimed invention. Therefore, the Office has failed to make a *prima facie* case of obviousness, and the present rejection should be withdrawn.

Obviousness Over Apple in View of Trau

Claims 1, 46 and 57-60 were rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Apple in view of Trau et al. (*Anal. Chem.* 2002, 74:3168-3173, hereinafter "Trau").

The teachings of Apple and the legal standard for obviousness have been described above. The Office acknowledges that Apple does not teach using an immobilization control probe and assessing the efficiency of immobilization probe, or using a labeled synthetic probe and the ratio between the hybridization signal involving the positive hybridization control probe and the hybridization signal involving the negative hybridization control probe and criteria for determining the positive signals. To cure these deficiencies of Apple, the Office cites Trau, which allegedly teaches a chemically modified and/or fluorescently labeled immobilization control probe on a chip, and further teaches that immobilization efficiency can be assessed by analyzing a signal from the immobilization control probe. The Office further alleges that Trau teaches determining the ratio of signal intensities and establishing criteria for the positive signal for the closely related probes. The Office argues that it would have been obvious to one of ordinary skill in the art at the time the invention was made to modify the HLA typing and data analysis of Apple to include an additional immobilization control probe and hybridization analysis method of Trau with the expected benefit of improving quantitative microarray data by normalizing hybridization data for each spot on the

chip in relation to the amount of immobilized probe thereby reducing the spot-to-spot variation due to unequal immobilization as taught by Trau.

Applicants respectfully traverse this rejection for substantially the same reasons as those set forth above. Neither Apple nor Trau nor a combination thereof teaches or suggests using a chip comprising all four immobilized control probes, or a chip comprising multiple immobilized positive control probes, wherein the variations in the length and sequence of said probes, when hybridized with the target nucleotide sequence or another nucleotide sequence in the preparation, create a group of hybridization signals having strong-to-weak or weak-to-strong orderly magnitude. In the absence of a teaching or suggestion of each and every claim element, the cited combination fails to provide the motivation to practice the presently claimed invention. Therefore, the Office has failed to make a *prima facie* case of obviousness, and the present rejection should be withdrawn.

Obviousness Over Apple in View of Samartziduo and Trau

Claims 1 and 47 were rejected under 35 U.S.C. § 103(a) as allegedly being unpatentable over Apple in view of Samartziduo and further in view of Trau.

The teachings of Apple, Samartziduo and Trau, and the legal standard for obviousness have been discussed above. The Office acknowledges that Apple does not teach using a negative control probe, a hybridization control probe and an immobilization control probe along with a positive control probe. To cure these deficiencies of Apple, the Office cites both Samartziduo, which allegedly teaches a chip that includes positive and negative hybridization control probes and probes for dynamic range and ratio controls, and Trau, which allegedly teaches using an immobilization control probe on a chip. The Office argues that it would have been obvious to one of ordinary skill in the art at the time the invention was made to modify the HLA typing method of Apple and include the hybridization control probe of Samartziduo and immobilization control probe and hybridization analysis method of Trau with the expected benefits of using a better controls for validation of microarray experiments, allowing assessment of target attachment, hybridization uniformity, detection limits, dynamic range and expression ratio as taught by Samartziduo, and improving quantitative microarray data by normalizing hybridization data for each spot on the chip

in relation to the amount of immobilized probe thereby reducing the spot-to-spot variation due to unequal immobilization as taught by Trau.

Applicants respectfully traverse this rejection. As discussed previously, claim 47 has been canceled, and its limitation has been incorporated into claim 1, thus rendering any comments directed to claim 47 moot.

Assuming, *arguendo*, that the combination of Apple, Samartziduo and Trau does teach a chip with positive, negative, hybridization and immobilization controls immobilized thereon, neither Apple nor Samartziduo nor Trau nor a combination thereof teaches or suggests using a chip comprising multiple immobilized positive control probes, wherein the variations in the length and sequence of said probes, when hybridized with the target nucleotide sequence or another nucleotide sequence in the preparation, create a group of hybridization signals having strong-to-weak or weak-to-strong orderly magnitude. It is important to recognize that the dynamic range controls and ratio controls disclosed in Samartziduo are not equivalent to the multiple positive controls claimed herein. In Samartziduo, the dynamic range controls and ratio controls are *in vitro* transcribed YIR mRNAs represented at specified concentrations and ratios, which are added to the mRNA sample before labeling (*see* page 1, right column). In the present application, however, all the control probes, including the multiple positive controls of different length and sequence, are immobilized on the chip, such that no additional controls need to be added along with the test sample. In the absence of a teaching or suggestion of each and every claim element, the cited combination fails to provide the motivation to practice the presently claimed invention. Therefore, the Office has failed to make a *prima facie* case of obviousness, and the present rejection should be withdrawn.

CONCLUSION

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to withdraw the outstanding rejection of the claims and to pass this application to issue. If it is determined that a telephone conference would expedite the prosecution of this application, the Examiner is invited to telephone the undersigned at the number given below.

In the event the U.S. Patent and Trademark office determines that an extension and/or other relief is required, applicant petitions for any required relief including extensions of time and authorizes the Commissioner to charge the cost of such petitions and/or other fees due in connection with the filing of this document to **Deposit Account No. 03-1952** referencing docket No. 514572001200. However, the Commissioner is not authorized to charge the cost of the issue fee to the Deposit Account.

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REVIEW

Progress in high throughput SNP genotyping methods

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ABSTRACT
Most current single nucleotide polymorphism (SNP) genotyping methods are still too slow and expensive for routine use in large association studies with hundreds or more SNPs in a large number of DNA samples. However, SNP genotyping technology is rapidly progressing with the emergence of novel, faster and cheaper methods as well as improvements in the existing methods. In this review, we focus on technologies aimed at high throughput uses, and discuss the technical advances made in this field in the last few years. The rapid progress in technology, in combination with the discovery of millions of SNPs and the development of the human haplotype map, may enable whole genome association studies to be initiated in the near future.
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Keywords:

INTRODUCTION

Large-scale association studies by genotyping many single nucleotide polymorphisms (SNPs), in individuals with well characterized phenotypes, are considered as promising methods to identify the cause of many complex diseases, or for improved understanding of the basis of variable response to drugs (pharmacogenetics). Ideally, such studies should be free of biological hypotheses and be done at the whole genome level, to maximize the likelihood of success. The recent completion of human genome sequencing and the discovery of several million SNPs have, for the first time, opened the door for genome-wide association studies. However, current genotyping technologies are too inefficient and expensive for whole genome analysis, which needs to analyze at least tens of thousands of SNPs in a large number of samples. This contrasts with gene expression analyses, where DNA chip/microarray-based methods have already made whole genome analyses possible. However, genotyping technologies are currently evolving rapidly, increasing the throughput and reducing the cost, as described in many recent reviews.[1–6] These improvements in genotyping methods will increase the feasible size of association studies, and may eventually allow whole genome-scale analyses.

In this review, we define 'high throughput genotyping' as genotyping of many SNPs (hundreds or more) in many individuals (hundreds to thousands), and especially focus on the technologies aimed at large-scale association studies. Such high throughput methods include Taqman, single-base extension-based assays, MALDI-TOF mass spectrometry-based systems, the Invader assay, Pyrosequencing and others. We will discuss the status of these technologies and their future potential. However, due to the limitation of space, we will not describe other types of 'high throughput' genotyping methods that are suited for typing a few SNPs in a large number of individuals, or typing a vast number of SNPs in a few individuals.

PCR-FREE GENOTYPING METHODS

Except for the classic restriction fragment length polymorphism (RFLP) analysis using Southern blotting, the vast majority of the SNP genotyping methods rely on pre-amplification of the SNP-containing genomic region by the polymerase chain reaction (PCR). The development of PCR techniques truly revolutionized genomic analyses including genotyping, by making a specific genomic region amenable to direct biochemical detection. In contrast to the yeast genome, where direct genotyping using genomic DNA by allele-specific hybridization is feasible,[7] the high complexity of the human genome makes such an approach very difficult. Thus pre-amplification by PCR has been necessary for most of the genotyping assays, despite the fact that the PCR process is relatively expensive, and is also challenging for automation because of the thermocycling steps.

There have been a few notable efforts to establish PCR-free genotyping methods. One such attempt is the Invader method[8] (Third Wave Technologies), based on a matched nucleotide-specific cleavage by a structure-specific 'flap' endonuclease, in the presence of an invading oligonucleotide. The clever combination of this reaction with a secondary reaction using fluorescence resonance energy transfer (FRET) oligonucleotide cassettes, generates a highly allele-specific signal, in a completely homogeneous and isothermal reaction ('Homogenous reaction' here is defined as a 'single vessel reaction' without involving a transfer between vessels). The whole process of this genotyping method is easily automatable. In addition, the Invader assay's great sensitivity and excellent signal to noise ratio allow direct genotyping of genomic DNA samples without PCR.[9] However, the amount of DNA currently required for reliable genotyping is too high (≥ 50 ng range) for the analysis of a large number of SNPs. The Invader method can be combined with PCR to reduce the DNA requirement, which also makes the signal more robust.[10,11] Another type of PCR-free genotyping is available through the combination of padlock probe ligation,[12] and signal amplification by the rolling circle DNA amplification (RCA) process.[13–17] In this assay, allele discrimination is accomplished by the specific ligation of completely matched oligonucleotides, in the same way as oligonucleotide ligation assay (OLA).[18] The difference here is that the ligation of a padlock probe creates a circular DNA, which can be amplified by rolling circle DNA synthesis by a DNA polymerase. The high degree of signal amplification by rolling circle synthesis and the specificity of the allele-discrimination by DNA ligase, make padlock probe/RCA assay sensitive enough to be directly applied to genomic DNA. However, typical padlock probe/RCA genotyping still requires a large quantity of DNA (100 ng) per genotype, again making it less than ideal for the analysis of many SNPs. A recent publication using FRET primers (Amplifluor) for signal detection[19] shows promise in reducing the DNA requirement to a nanogram level, but it still needs to be tested if this modification can be generalized to many different SNPs.

In summary, these two 'PCR-free' methods are less than ideal for high throughput analysis of a large number of SNPs today, because of their requirement of a large quantity of genomic DNA. While the improvements in these technologies may reduce the needed DNA amount, they still are likely to require more DNA than PCR-based assays. However, they may still make competitive high throughput genotyping platforms, if they are combined with a suitable pre-amplification step. The Invader assay in fact, has been successfully combined with PCR.[10,11] In the latter, an ultra high throughput set up is created by combining multiplex PCR and a miniaturized single-plex Invader reaction; 300 000–400 000 genotypes have been reported to be scored in a day using this method.[11]

SINGLE-STEP HOMOGENEOUS METHODS

Taqman,[20] molecular beacon,[21] and scorpion assay[22,23] are all microtiter plate-based fluorescent readout systems, initially designed for real time PCR expression analyses. Taqman and molecular beacon both rely on allele-specific hybridization of oligonucleotides during PCR for allele discrimination, while scorpion assay can use either allele-specific PCR[23] or allele-specific hybridization[22] chemistry for allelic discrimination. They all can be performed as an endpoint assay in a completely homogeneous reaction. All the reagents and genomic DNA are mixed at the beginning, and the fluorescent signal is read after the thermocycling step. There is no separate pre-amplification step, or intermediate processing, making them the simplest assay formats possible. The lack of a 384-well fluorescence detector suited for these methods, the cost of fluorescently labeled probes, and the lack of a reliable automated genotype calling software have been some of the issues hindering these methods. The recent release of the 7900HT real-time PCR machine from Applied Biosystems has made a 384-well format assay possible, contributing to a higher throughput and a reduced reaction volume. Probe cost becomes less of an issue when many thousands of DNA samples are analyzed in a reduced reaction volume. In the case of Taqman, the current automated genotype calling software for the 7900HT machine relies on positive controls, and when they don't exist in the same plate, manual genotype calling upon visual data inspection is required. A reliable automated allele calling capability is essential when the genotyping throughput becomes higher, not just to make correct genotype calls faster, but also to process and track the data quickly and accurately. A recent study demonstrated the feasibility of using a cluster analysis for automated genotype calling without using positive controls.[24] Since the Taqman assay often gives background fluorescent signals, the development robust and reliable allele-calling algorithm based on such cluster analyses is especially important.

There are two notable recent additions to the list of single-step homogeneous genotyping systems. One is an allele-specific PCR-based assay using tailed allele-specific amplification primers that have secondary priming sites for universal energy-transfer-labeled primers.[25] Another is the AlphaScreen proximity assay.[26] AlphaScreen generates an amplified light signal when donor and acceptor beads are brought to proximity, and this detection method can be

combined with allele-specific amplification chemistry or allele-specific hybridization chemistry for allele discrimination. Both of these methods provide simple assay formats and are relatively cost-effective because the same set of fluorescently labeled primers can be used for many different SNPs.

These methods provide one of the highest throughput today, reaching tens of thousands of genotypes a day. However, multiplex assays are not currently available, limiting its potential for an even higher throughput. This lack of multiplexing capability is largely due to the difficulty in discriminating the signals from different dyes with overlapping spectra, limiting the number of compatible dye combinations. Progress in dye chemistry allowing a high degree of multiplexing and miniaturization will dramatically improve the throughput and cost efficiency of these methods. (Some of the progress in dye chemistry is discussed below.)

HOMOGENEOUS DETECTION WITH FLUORESCENCE POLARIZATION

Fluorescence polarization detects the increase in polarization of fluorescence, caused by the decreased mobility of a fluorophore through a molecular mass increase. When this detection method is combined with the single-base extension assay (SBE, also called genetic bit analysis; GBA, or mini-sequencing), the allele-specific incorporation of fluorescently labeled dideoxy-NTP can be detected as an increase of a polarized fluorescence.[27] This method provides a relatively inexpensive (no need for fluorescently labeled primers) and simple detection platform for genotyping. Fluorescence polarization detection can also be used in combination with other allele-discrimination chemistries, such as allele-specific PCR,[28] Taqman[29] and Invader.[30] Fluorescence polarization platform may be more suited for medium throughput genotyping (hundreds to low thousands a day), rather than for a higher throughput, because of the lack of multiplexing capability. It also has a disadvantage of being a multi-step process (PCR and post-PCR processing steps), compared to single-step methods described in the previous section. It also has a relatively modest signal/noise ratio, as the incorporation of a fluorescently-labeled nucleotide into the primer end changes its molecular weight only by an order of magnitude, which makes a reliable automated allele calling in this method a challenge. Addition of *E. coli* single-strand DNA binding protein (SSB) to increase the apparent size of the primer to improve this signal/noise ratio has been designed to address this issue.[31] This method requires more involved steps than the single step homogeneous methods, and may not generate the highest throughput in the future. Nevertheless, it is still a very reasonable choice for a laboratory needing a convenient and relatively cost-effective genotyping method.

PYROSEQUENCING

Pyrosequencing[32-34] employs an elegant cascade of enzymatic reactions to detect nucleotide incorporation during DNA synthesis. When a nucleotide is incorporated at the 3'-end by DNA polymerase, a pyrophosphate is released that is immediately converted to ATP by ATP sulfurylase. This ATP causes the oxidization of luciferin by luciferase, which is detected as a light signal. Pyrosequencing was initially developed as a DNA sequencing method, with a chemistry completely different from the Sanger dideoxynucleotide method. It is also a unique homogeneous sequencing method with no electrophoresis. However, Pyrosequencing has a limited use in *de novo* DNA sequencing, because of the relatively short read length. On the other hand, its capability to read flanking sequences as well as the SNP position itself, and its high specificity (ie non-specific binding will not generate a false signal) make it an accurate and attractive SNP genotyping method. In this method, alleles can be called by analyzing the individual sample itself, without comparing its signal to that of other samples or controls. This makes Pyrosequencing suitable for fully automated genotype calling, an important component of high throughput analyses. A 96-well medium throughput machine and a fully automated 384-well format high-throughput machine, are already available from Pyrosequencing AB (Uppsala, Sweden) for this method, and the latter has capacity to score high thousands to low tens of thousands of genotypes a day. Pyrosequencing can be done in a duplex or a triplex format at least for some SNP combinations. However, any higher degree of multiplexing is likely to be difficult, limiting the potential for future throughput increase.

DNA CHIP/ARRAY BASED ASSAYS

When the DNA chip (oligonucleotide or DNA attached on solid phase) technology first appeared, it provided a great hope as a highly parallel genotyping method using a simple allele-specific hybridization detection.[35-37] However, while the DNA chip technology completely revolutionized the RNA expression analysis field,[38] and also has been used for SNP discovery,[39-41] DNA chip based allele-specific hybridization genotyping has seen a rather limited number of uses so far.[42-44] This is mainly due to the difficulty in obtaining a good signal/noise ratio in allele-specific hybridization. The specificity of discrimination between completely matched and mismatched oligonucleotides in hybridization is limited, and is much lower than that of enzymatic discrimination using DNA polymerases or DNA ligases. This specificity problem especially worsens when many different oligonucleotides need to be hybridized under a single condition. This poor specificity compromises genotype call rates and accuracy. There is no easy solution for this problem, despite many efforts in the past, including the use of chips designed with high redundancies.[42-44] The use of nucleic acid chaperons[45,46] can potentially solve this problem, as they provide higher specificity for oligonucleotide hybridization, but so far they have yet to see any application in genotyping.

Combination of single-base extension with solid-phase bound oligonucleotides[47-49] has been successfully applied to microarray formats for parallel SNP analysis.[50,51] These methods give much more specific signal than allele-specific hybridization based platforms, because of the high accuracy of DNA polymerases in incorporating complementary

nucleotides during base extension. Single-base extension is a robust chemistry that can be used with homogeneous and electrophoresis detection methods.[27,52] The SNP to SNP variation of the optimal reaction condition is relatively small in single-base extension reactions, making it suitable for multiplexing. One variation of this method is to incorporate allelic variation at the 3′-end of primers to detect allele-specific extension.[53] Allele-specific extension platform has advantages in simplified handling of multiple samples, as well as in requiring only one or two fluorophores per reaction.

There also are certain disadvantages in these solid phase single-base extension or allele-specific primer extension formats: (1) These platforms require SNP-specific primers to be attached on a solid surface, making them rather inflexible (needing different DNA chips to analyze different SNPs), and limited in cost efficiency. (2) A solid phase reaction is generally less efficient, due to a lack of diffusion of primer oligonucleotides. (3) It requires the 3′-OH terminus of the primer to be free and exposed, because this is where dideoxynucleotides are attached by DNA polymerases. Some DNA chips such as Affymetrix's GeneChip, in which the 3′-end of the primer is attached to the solid surface, are not suitable for this method because of this.

'Tag' based genotyping methods have been developed to overcome some of these limitations in the solid-phase reaction methods.[54,55] In this system, a highly multiplexed single-base extension reaction is performed in a solution using primers containing a 'tag' sequence at the 5′-end, and fluorescently labeled dideoxynucleotides. The 'tag' sequences in the primer are not involved in the single-base extension reaction itself, but are later used for these oligonucleotides to be captured on the surface of the DNA chip. After allelic discrimination by single base extension, the extended primers are captured through hybridization between tags and complementary oligonucleotides on the DNA chip. The advantages of such 'tag' array methods over solid phase single-base extension methods are the following: (1) generic DNA chips can be used, which contribute to cost reduction and flexibility; (2) enzymatic reaction is performed in the solution; (3) less restriction on the DNA chip formats, because there is no need for a free 3′-OH residue at the oligonucleotide end.

The word 'flexibility' here refers to the capability to use the same DNA chip format for different SNP genotyping assays. This flexibility is important because there is no clear consensus for which exact SNPs should be genotyped in the whole genome analysis. Creating a 'haplotype map' for the human genome is one way to select a smaller number of SNPs that can be used commonly for whole genome association studies.[56] However until such a 'common use SNP' set is established, different studies are likely to require genotyping different sets of SNPs, making a 'flexible' format more desirable.

The usefulness of generic DNA tag arrays was first demonstrated in parallel quantification of bacterial and yeast deletion mutants.[57,58] The first genotype application of this concept actually employed oligonucleotide ligation reaction (also called ligation detection reaction) as the allele-discrimination method.[59] Ligation-based chemistry offers a certain advantage for multiplexing as it has less interference between primers than base extension based assays. (Multiplexed single-base extension reaction has a potential of increased background from a false extension of other primers in the solution, causing a limit in multiplexing capability.) Another potential advantage is that the ligation-based assays have no need for post-PCR removal of carryover dNTPs. It is also well suited for detecting low abundance mutations in heterogeneous sources such as tumor tissues.[59,60] However, the ligation-mediated detection methods tend to be more expensive than single-base extension, because of the cost of SNP-specific fluorescent primers. Single-base extension reaction, on the other hand, can use a common set of fluorescent dideoxynucleotides for any SNP. Using degenerate fluorescent oligonucleotides for ligation has been shown to be a viable and cheaper alternative to the use of SNP specific fluorescent oligonucleotides.[61] Addition of a secondary detection system, such as rolling circle reaction after ligation is also tested with good results.[60] In the long term, this approach may be advantageous over the use of fluorescent primers, because of the potential for cost reduction and increased signal strength.

In conclusion, the combination of 'tag' array and single-base extension or ligation-based enzymatic reactions provides genotype platforms with ultra high throughput potential. Companies such as Orchid Bioscience, Applied Biosystems, and Affymetrix have been developing such 'tag-array' based genotyping systems.

BEAD-BASED METHODS

The concept of these assays is very similar to that of DNA chip based assays. However, oligonucletides are attached to small microspheres (3–5 μm in diameter) rather than to a fixed surface of DNA chips in these platforms. Bead-based systems can be combined with most of the allele-discrimination chemistry used in DNA chip based 'tag' array assays, such as single-base extension and oligonucleotide ligation assay. The bead-based format has great flexibility for multiplexing and SNP combination. In bead-based assays, the identity of each bead needs to be determined, and that information will be combined with the genotype signal from the bead to assign a genotype call to each SNP and individual. For this reason, it is essential to have some type of 'molecular identification' on each of the beads.

One of the bead-based genotyping platforms uses fluorescently coded microspheres developed by Luminex.[62] These beads are coated with two different dyes (red and orange), and can be identified and separated using flow cytometry, based on the amount of these two dyes on the surface. By having a hundred types of microspheres with a different red:orange signal ratio, it is possible to perform a hundred-plex detection reaction in a single tube. After the reaction, these microspheres are distinguished using a flow fluorimeter, and a genotyping signal (green) from each group of microspheres is measured separately. In essence, each tube becomes equivalent to a DNA chip with a hundred pos-

itions. Now with the availability of 96-well flow fluorimeters, it is technically possible to score thousands of genotypes in a single 96-well format reaction. This bead-based platform has been shown to work with allele-specific hybridization,[63] single-base extension,[64,65] allele-specific primer extension,[66] and oligonucleotide ligation assay.[61] Even though this system already achieves a reasonably high throughput, there are a few factors that might prevent this system from achieving even higher throughput. The number of currently available combinations of dye quantities for bead identification sets the limit at 100-plex. Another limitation is the number of dyes that can be used for the genotyping signal. With red and orange channels already dedicated for bead identification, actually only one color (green) is currently available for the genotyping signal detection, limiting the possibility of further multiplexing.

In a different bead-based platform commercialized by Illumina, microspheres are captured in solid wells, which were created from optical fibers.[67,68] The diameter of each well is similar to that of the spheres, allowing only a single sphere to fit in one well. Once the microspheres are set in these wells, all of the spheres can be treated like one high-density microarray. However there is a major difference. Because it is not known *a priori* which sphere is in which well, a 'decoding' process is needed to identify each bead after it is immobilized. In contrast to the Luminex system above, the genotyping reaction is performed after the beads are immobilized in this platform. In this sense, the Illumina bead system is more similar to a DNA chip platform rather than to a suspension array system. This 'decoding' process is accomplished by serial hybridization against fluorescently labeled 'decoding' oligonucleotides, and both 'decoding' and genotype signal reading involve a 'tag' hybridization. In this method, ~50 000 microspheres can be assembled in a single array. These properties give the fiber optics-based bead array system an extremely high multiplexing potential. So far, the only published genotyping application of this technology used molecular beacon as the allelic discrimination chemistry,[68] but this optic-fiber detection system should also work well with other allele-discrimination chemistries. In fact, Illumina recently started a genotyping service based on oligonucleotide ligation assay. Its multiplexing capability, ease of handling, and cost-effective manufacturing process offer a great potential for ultra high throughput genotyping using this method. This assay is currently only available as a service by Illumina, but Applied Biosystems and Illumina are co-developing equipment and reagent kits for this platform.

PROGRESS IN THE FLUORESCENT DYE CHEMISTRY

Improvements in fluorescent dye chemistry will have an impact on all the genotyping technologies using fluorescence detection, including homogeneous methods, DNA chip and bead-based systems, by increasing their multiplexing capabilities. For example, quantum dot dyes[69,70] are brighter, are more stable against photo bleaching, and have much narrower spectra linewidth than conventional fluorescent dyes, making them ideal for highly multiplexed assays. Development of assays using such dyes can greatly contribute to an enhanced genotyping throughput in the future.

MASS SPECTROMETRY BASED GENOTYPING ASSAYS

The principle of this method is to use mass spectrometry to detect the product of enzymatic allele-discrimination reaction directly or indirectly.[3,71,72] Various allele discrimination chemistries such as single-base extension and its variation,[73–82] allele-specific hybridization of peptide nucleic acid (PNA),[83–85] Invader,[86] and allele-specific PCR,[87–89] have all been successfully combined with the mass spectrometry detection. Combinations of single-base extension or its modifications with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry are the most commonly used, and have been made into commercial products by companies such as Sequenom and Applied Biosystems/PerSeptive Biosystems.[73–75,79,81,82] (The product from Applied Biosystems/Perseptive Biosystems is no longer available for this use currently.) The advantage of the MALDI-TOF mass spectrometry-based detection is in its speed and multiplexing capability. For example, a moderate mass spectrometer capable of recording 40 000 spectra a day, can theoretically score 200 000 genotypes in a 5-plex detection format. However, their rate limiting steps are generally not in the detection process by a mass spectrometer, but are in the preceding enzymatic reactions, and post-reaction sample processing steps. In most mass spectrometry-based assays, 5-plex may be the realistic limit for multiplexing to get reliable signals, partly due to the limitations in the detectable mass range and in the sensitivity of mass discrimination. Post-reaction sample processing is more complicated than that of most other genotyping formats, as a very high purity is necessary for the samples to be analyzed by a mass spectrometer. Solid phase sample processing with ion-exchange resin is employed in Sequenom's MassArray automated system,[81,82] while miniaturized reverse phase liquid chromatography is used for Applied Biosystems/PerSeptive Biosystem's product[74,75] to address this issue. Another system called 'GOOD assay' involves a use of chemically modified primers in the reaction, followed by an enzymatic removal of unextended primers and alkylation of the product, allowing a simplified and effective sample preparation for mass spectrometry.[76,77]

Genotype accuracy due to the intrinsic nature of mass spectrometry is another advantage. The sensitivity of the instrument, the mass specificity of each reaction product, and for some type of reactions the fact that each reaction contains internal standards for calibration, all contribute to this accuracy. Mass spectrometry-based methods give little background especially when detecting the allelic discrimination reaction products directly, allowing accurate and automated genotype calling.

A different mass spectrometry-based assay has been made into a commercial product as Qiagen's MassCode system. This assay combines allele-specific PCR with UV-cleavable 'mass tags', and mass spectrometry detection.[87] Here, mass spectrometry detects the cleaved tags and not the extension

products themselves. Use of these 'mass tags' makes highly-multiplexed detection by a relatively simple mass spectrometer possible. One the other hand, this method can be more prone to background signal at least theoretically, as the mass spectrometer does not directly detect the allele-discrimination reaction product. For example, incomplete removal of free 'mass tag' labeled primers before UV-cleavage can cause a false signal in this method.

The throughput of mass spectrometry-based genotyping assays is among the highest in today's commercially available genotyping systems. For example, Sequenom's MassArray system can routinely score tens of thousands of genotypes a day. Mass spectrometry-based genotyping systems are certainly one of the main contenders for ultra high throughput genotyping in the future whole genome association studies.

CONCLUSION

There exist at least 20 different SNP genotyping methods today, consisting of various combinations of different allele-discrimination chemistries and signal detection methods. Many of these methods have been developed into commercial products with a 384-well format and automation, such as Orchid Bioscience's single-base extension-based SNPStream, Sequenom's MALDI-TOF mass spectrometry-based MassArray system, and Pyrosequencing's high throughput system obtaining a throughput of ≥10 000 genotypes a day.

Mass spectrometry-based assays, 'tag' array-based assays, and single-step homogeneous methods all have distinct advantages (multiplexing capability and good signal/noise ratio in mass spectrometry-based assays, extreme multiplexing capability in 'tag' array systems, and fast and simple processing in single-step homogeneous methods) for high throughput genotyping, and probably are the front runners to realize the potential of whole genome association studies. Some other methods described here such as Invader (combined with PCR), Pyrosequencing, and fluorescent polarization-based methods are also evolving fast to attain higher throughput. However, the lack of potential for a high level of multiplexing compared with mass spectrometry or 'tag' array-based methods, and extra handling steps compared with single-step homogeneous systems, are the challenges for these assays in obtaining the next level of throughput. Current non-PCR-based RCA assays need to solve the issue of high DNA quantity requirement before it becomes feasible for analyzing many SNPs. There also are other emerging methods such as dynamic allele-specific hybridization (DASH).[90,91] The DASH assay achieves a cost-effective genotyping in a simple handling, but it still needs to be seen if this will evolve into a competitive high throughput platform with a progress of equipment.

Today's cutting edge technologies are capable of tens of thousands of genotypes per day with automation, making large-scale association studies possible. Whole genome association analyses, however, still need a higher throughput. Haplotype block information for the entire genome will enable us to choose a set of the most informative SNPs, helping to reduce the number of SNPs needed to be genotyped for whole genome association studies. However, it will still be necessary to genotype tens of thousands of SNPs at the very least, because the sizes of haplotype blocks in the human genome appear to be relatively small, in the range of tens of kilobases or less.[92–94] Whole genome association analysis using 1000 samples and 100 000 SNPs in one year, will need a throughput of >300 000 genotypes a day, which is still one order of magnitude higher than what is available today. Nevertheless, this is probably attainable in the near future. At least, this throughput is theoretically already achievable by running 10 or more of today's high throughput genotyping platforms. It is probably not too optimistic to assume that a few whole genome association studies will be completed in the next few years, with the recent technological progress in this field.

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